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Fas (CD95) expression and death-mediating function are induced by CD4 cross-linking on CD4$^+$ T cells

[programmed cell death/HIV/*lpr* (lymphoproliferative)]

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ABSTRACT **The CD4 receptor contributes to T-cell activation by coligating major histocompatibility complex class II on antigen presenting cells with the T-cell receptor (TCR)/CD3 complex, and triggering a cascade of signaling events including tyrosine phosphorylation of intracellular proteins. Paradoxically, CD4 cross-linking prior to TCR stimulation results in apoptotic cell death, as does injection of anti-CD4 antibodies *in vivo* or CD4 ligation by HIV glcoprotein (gp) 120. In this report we investigate the mechanism by which CD4 cross-linking induces cell death. We have found that CD4 cross-linking results in a small but rapid increase in levels of cell surface Fas, a member of the tumor necrosis factor receptor family implicated in apoptotic death and maintenance of immune homeostasis. Importantly, CD4 cross-linking triggered the ability of Fas to function as a death molecule. Subsequent to CD4 cross-linking, CD4$^+$ splenocytes cultured overnight became sensitive to Fas-mediated death. Death was Fas-dependent, as demonstrated by cell survival in the absence of plate-bound anti-Fas antibody, and by the lack of CD4-induced death in cells from Fas-defective lymphoproliferative (*lpr*) mice. We demonstrate here that CD4 regulates the ability of Fas to induce cell death in CD4$^+$ T cells.**

The CD4 coreceptor and the T-cell receptor(TCR)/CD3 complex on T cells bind peptide–major histocompatibility complex class II complexes on antigen presenting cells during antigen specific immune responses (1). CD4 contributes to T-cell activation by signaling via its associated *src*-family tyrosine kinase, p56^{lck} (lck), which phosphorylates the CD3 γ, ε, and ζ chains (2). CD4 coengagement with the TCR facilitates T-cell activation. In contrast, CD4 cross-linking prior to activation via the TCR results in apoptotic cell death (3). CD4 cross-linking by HIV glycoprotein (gp) 120 also results in apoptosis (4). Furthermore, *in vivo* CD4 ligation by injection of anti-CD4 antibodies triggered loss of surface CD4 expression, and DNA fragmentation and death (5). *In vivo* CD4-induced apoptosis appeared to be Fas (CD95)-dependent, since it did not occur in MRL-*lpr/lpr* mice, which have a mutation in the Fas gene resulting in a nonfunctional protein.

Fas, a member of the tumor necrosis factor receptor family, is a mediator of cell death (6). Fas–Fas ligand (FasL) interactions mediate activation-induced cell death, the apoptotic death which follows TCR/CD3-induced T-cell activation (7–9). *In vivo*, Fas/FasL are thought to be responsible for controlling clone size after expansion in responses to peptide antigens and superantigens, and thus are critical in the regulation of immune homeostasis (10, 11). Fas knockout mice and mice with natural mutations in the Fas or FasL genes, *lpr* (lymphoproliferative) and *gld* (generalized lymphoproliferative defect), respectively, develop massive lymphadenopathy that becomes worse with age (12–14).

In this report we demonstrate that CD4 cross-linking induces a small but rapid increase in cell surface Fas expression on isolated CD4$^+$ T lymphocytes. CD4 crosslinking also results in Fas-dependent cell death in normal and *gld*, but nor *lpr*, strains of mice. Finally, we show that CD4 cross-linking triggers a subset of CD4$^+$ cells to become sensitive to Fas-induced cell death, by a mechanism separate from control of Fas surface expression.

MATERIALS AND METHODS

Mice. Female mice 6–10 weeks of age were obtained from the The Jackson Laboratories. Mice of the inbred strains C3H, C3H-*lpr/lpr*, and C3H-*gld/gld* were used.

CD4$^+$ Cell Isolation. Single cell suspensions were prepared from murine spleens and depleted of erythrocytes with Gey's solution. CD4$^+$ cell isolation was accomplished by negative selection on commercial CD4 isolation columns (Biotex Laboratories, Edmonton, Canada). In normal mice, yields were 90% or more CD4$^+$ cells as determined by flow cytometry. In *lpr* and *gld* mutant mice, a larger percentage of CD4$^-$ cells were present due to the accumulation of CD3$^+$4$^-$8$^-$ cells (15), which could not be removed by the negative selection columns. However, CD4$^+$ cells were examined selectively by gating during flow cytometric analysis.

CD4 Cross-Linking. Cell suspensions were adjusted to 10^7 cells/ml in PBS supplemented with 5% fetal calf serum. Suspensions were incubated with 10 μg/ml biotinylated anti-CD4 antibody (protein G-purified, biotinylated GK1.5 monoclonal antibody) for 15 min at room temperature, washed twice in PBS, then cross-linked with streptavidin (Jackson ImmunoResearch) at 0.02 unit/μg antibody, as recommended by the manufacturer. Cells were incubated at 37°C for 45 min, then again washed twice. Control cells were treated with 10 μg/ml rat IgG or untreated as indicated, and then incubated with streptavidin as were the anti-CD4-treated cells.

Fas Expression. Surface levels of Fas expression after CD4 cross-linking were determined by flow cytometry. Cells were dual-stained with anti-Fas-fluorescein isothiocyanate (FITC) (clone Jo2; PharMingen) and anti-CD4-phycoerythrin (clone H129 or MW4; PharMingen). A total of 10,000–20,000 events were acquired for each sample on a FACScan (Becton Dickinson), and Fas expression was analyzed on gated CD4$^+$ populations unless otherwise indicated.

Cell Cultures. Purified CD4$^+$ cells or spleen cell suspensions (erythrocyte-depleted whole spleen) were cultured overnight (16–20 hr) in 96-well tissue culture plates at 5×10^5 cells/well

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Abbreviations: TCR, T-cell receptor; FasL, Fas ligand; FITC, fluorescein isothiocyanate.

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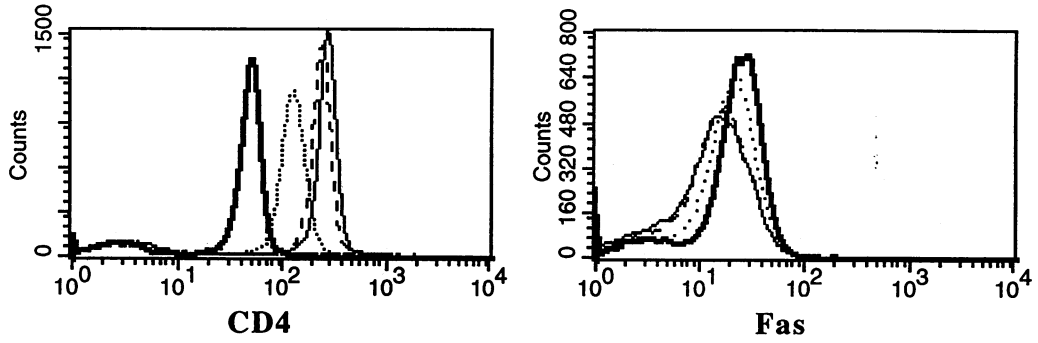

FIG. 1. CD4 cross-linking upregulates Fas expression within 45 min on isolated CD4+ cells. As anti-CD4 concentration is increased, CD4 is down-modulated (*Left*) and Fas cell surface expression increases (*Right*). Anti-CD4 was used at 0.1 μg/ml (dashed line), 1 μg/ml (dotted line), or 10 μg/ml (thick line). Uncross-linked cells are shown as a control (solid line).

in PBS with 5% fetal calf serum. Fas antibody ligation was accomplished by precoating the 96-well plates with anti-Fas antibody (Jo2). Plates were incubated with 10 μg/ml antibody, 100 μl/well in PBS, for 1 hr at 37°C, then washed three times in PBS prior to plating cells for overnight culture.

Determination of Surface Phenotype and Cell Death After Culture. After overnight culture, cells were washed and stained at 4°C with anti-CD4-FITC (GK1.5 prepared in our laboratory) or with anti-CD4-phycoerythrin and anti-Fas-FITC in the 96-well plates in which they were cultured. Cells were harvested on ice and analyzed by flow cytometry. Dead cells were discriminated according to light scatter and ethidium bromide staining. Light scatter analysis, previously described as a flow cytometric technique for the analysis of cell death (16), was performed by setting gates on forward versus side light scatter dot plots. Dead cells are smaller and more granular than live cells, consistent with the characteristic morphological determinants of apoptotic cell death, cell shrinkage and blebbing (17). An additional assay for cell death was carried out by adding ethidium bromide (Sigma) at a final concentration of 10 μg/ml to the cell suspensions immediately before acquiring the sample. Live cells exclude ethidium bromide, which intercalates into the DNA of dead cells. The techniques correlate within 3% for the determination of cell death. In addition, we have verified that in these types of experiments the percentage of cells staining with ethidium bromide correlates with the percentage of apoptotic cells determined by the TdT/dUTP-FITC method (Elizabeth Ward and David Wagner, personal communication). We have elected to use ethidium bromide staining and change in cell size and granularity as parameters for cell death to quantify total cell death (18).

RESULTS

CD4 Cross-Linking Rapidly Upregulated Fas Expression in Isolated CD4+ Cells. We have examined the effects of CD4 cross-linking on cell surface Fas expression in isolated CD4+ splenocytes (Fig. 1). After 45 min of cross-linking, mean Fas expression increased by more than two-fold. Maximal Fas expression, however, was not altered, despite the significant increase in population mean expression levels. Fas upregulation increased with increasing concentrations of anti-CD4 antibody used to cross-link (Fig. 1). No change in Fas expression was observed when cell surface CD3 was cross-linked for 45 min (Fig. 2), although CD3 stimulation for 16 hr upregulates Fas expression by more than 10-fold (data not shown). Short-term co-cross-linking of CD3 with CD4 has the same effect as cross-linking CD4 alone (Fig. 2). Thus, Fas upregulation is a rapid, specific response to CD4 cross-linking.

In all strains of mice examined, including C57BL/6, BALB/c, AKR/J, NZB × NZWF1, and C3H/HeJ, a significant increase in cell surface Fas expression was observed within 45 min of CD4 cross-linking, although the initial level of Fas expression per cell was strain dependent (data not shown). In mice bearing the *lpr* mutation, basal Fas expression is very low (19); nonetheless, an increase in Fas expression was observed after CD4 cross-linking, suggesting that CD4-induced Fas upregulation occurs independently of the ability of Fas to induce death (Fig. 2*B*). CD4+ lymphocytes from *gld*

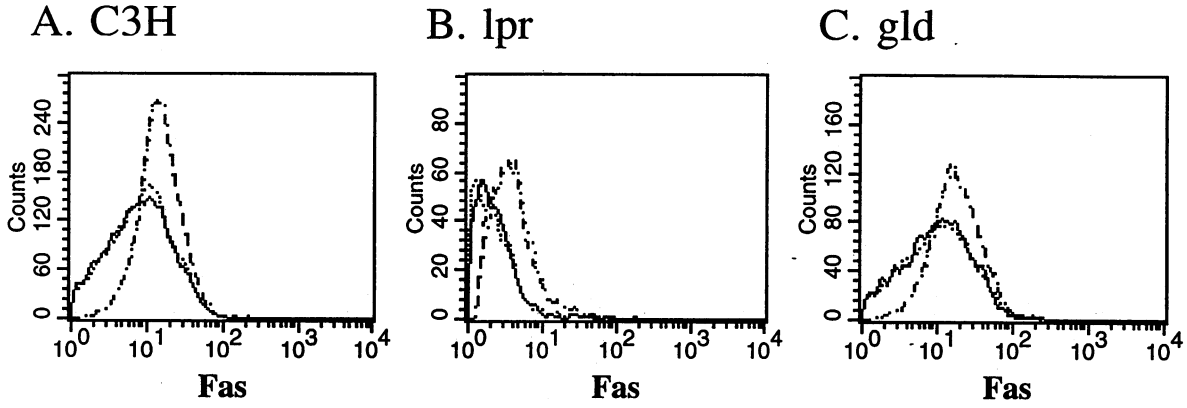

FIG. 2. CD4-induced Fas upregulation is not affected by CD3 cocross-linking, and is independent of functional Fas and FasL. Isolated CD4+ T cells which were not cross-linked (solid lines) or were anti-CD3 cross-linked (dotted lines) expressed identical levels of Fas. Cells which were cross-linked with anti-CD4 alone (dashed lines) or were cocross-linked with anti-CD4 and anti-CD3 (spaced dotted lines) upregulated their Fas expression to identical levels. Fas upregulation in response to anti-CD4 and anti-CD4 plus anti-CD3 cross-linking occurred in wild-type C3H mice (*A*), C3H-*lpr*/*lpr* mice whose Fas function is defective (*B*), and FasL-defective C3H-*gld*/*gld* mice (*C*).

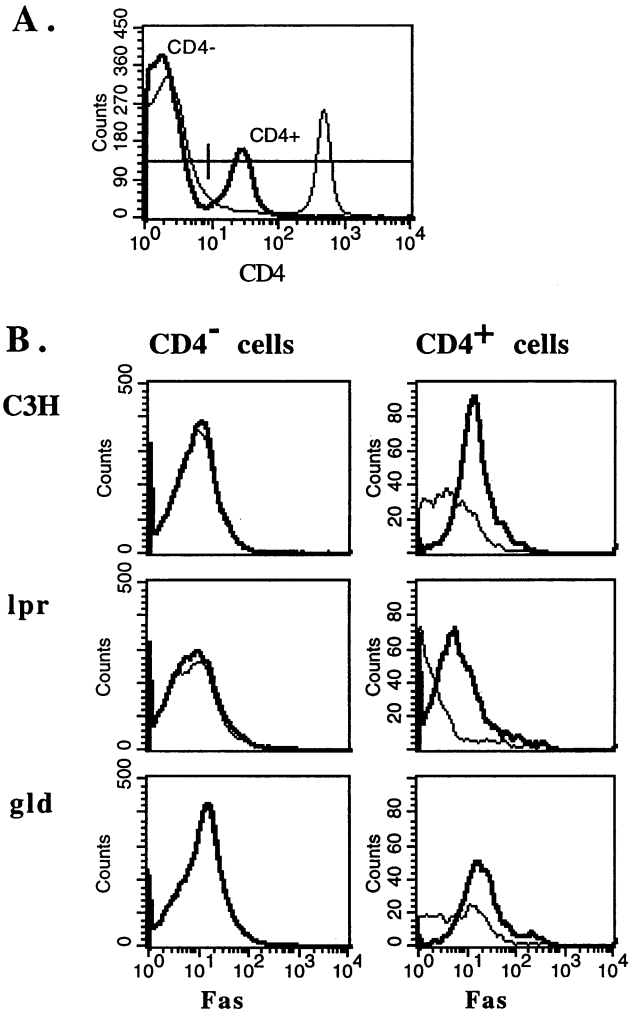

FIG. 3. Fas upregulation in response to CD4 cross-linking of unfractionated splenocytes occurs in CD4$^+$, but not CD4$^-$, cells. (*A*) Analysis gates were set to examine CD4$^+$ and CD4$^-$ populations in unfractionated spleen cultures which were uncross-linked (solid lines) or cross-linked with anti-CD4 (thick lines). (*B*) Fas expression on CD4$^-$ and CD4$^+$ uncross-linked (solid lines) and cross-linked (thick lines) splenocytes are shown. Fas expression is upregulated only on CD4$^+$ cells after CD4 cross-linking.

mice also upregulate Fas, demonstrating that this mechanism is independent of functional FasL (Fig. 2*C*).

CD4 Cross-Linking Rapidly Upregulated Fas Expression in CD4$^+$, But Not CD4$^-$, Cells in Whole Spleen. It has been reported that cytokines such as tumor necrosis factor α can upregulate Fas in CD4$^+$ and CD8$^+$ cells after several days of culture (20). To determine whether the presence of other cell types affected CD4-induced Fas upregulation, we treated whole spleen suspensions with the CD4 cross-linking protocol (Fig. 3). CD4$^+$ lymphocytes in the suspension displayed Fas upregulation identical to that of isolated cells, and CD4$^-$ cells underwent no change in Fas expression. These results demonstrate that there are no paracrine effects on Fas surface expression between CD4$^+$ and CD4$^-$ cells.

CD4 Cross-Linking Resulted in Increased Fas-Dependent Cell Death During Overnight Culture. We examined the ability of Fas to induce death by determining the extent of Fas-dependent cell death following overnight culture of isolated CD4$^+$ splenocytes and whole spleen suspensions. Fas dependent death was assessed by comparing cultures of C3H*wt* versus C3H-*lpr*/*lpr* cells incubated with and without plate bound anti-Fas antibody. Our results indicate that in isolated

Table 1. CD4-induced, Fas-dependent cell death during overnight culture of isolated CD4$^+$ cells

Mouse strain	CD4 cross-linked*	% live cells − Anti-Fas Ab$^+$	% live cells + Anti-Fas Ab$^+$	Fas-dependent increase in cell death, %
C3H	−	47.9 ± 4.8	44.5 ± 3.0	3.3
	+	52.5 ± 4.5	31.6 ± 3.7	20.6‡
C3H-*lpr*/*lpr*	−	22.4 ± 2.9	22.7 ± 3.6	−0.26
	+	20.0 ± 1.5	20.3 ± 0.7	−0.32

Cell death was determined by EtBr incorporation.
*Prior to overnight culture, cells were CD4 cross-linked for 45 min (+) or sham treated (−).
†Anti-Fas antibody was coated onto wells prior to overnight culture.
‡Fas treatment makes a significant difference ($P < 0.02$ by Student's *t* test).

CD4$^+$ splenocyte cultures, the percentage of cell death increases significantly only when the cells have been cross-linked with anti-CD4 and incubated overnight with anti-Fas antibody (Table 1). Thus, CD4 cross-linking activates the ability of the Fas molecule to induce death, as well as upregulates its expression. In *lpr* mice, CD4 cross-linking does not cause an increase in cell death, suggesting that the cell death triggered by CD4 cross-linking in normal mice is entirely Fas-dependent. It should be noted that the spontaneous death during overnight culture of *lpr* lymphocytes (both B and T) is higher than for normal lymphocytes, for reasons not yet understood (21).

We have observed similar results in unfractionated spleen cultures (Fig. 4). Again, Fas acquires the ability to mediate death as a consequence of CD4 cross-linking in a subset of CD4$^+$ cells (Fig. 4*A*). In contrast, CD4$^-$ cells are not sensitized to Fas-mediated death (Fig. 4*B*).

Fas Function Is Regulated by CD4 Cross-Linking Independently of the Cell Surface Level of Fas Expression. We examined whether the CD4-induced increase in Fas-mediated cell death was simply a consequence of the small increase in cell surface Fas expression or whether CD4 cross-linking also

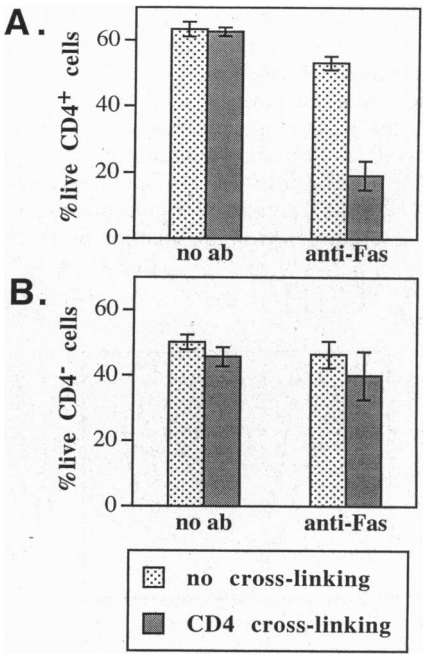

FIG. 4. CD4 cross-linking induces Fas-dependent cell death in CD4$^+$, but not CD4$^-$, splenocytes during overnight culture. The percentage of live cells after overnight incubation with or without plate-bound anti-Fas antibody, following CD4 cross-linking or control rat IgG treatment, is shown. Flow cytometry analysis gates were set on CD4$^+$ cells (*A*) and CD4$^-$ cells (*B*) as shown in Fig. 3*A*.

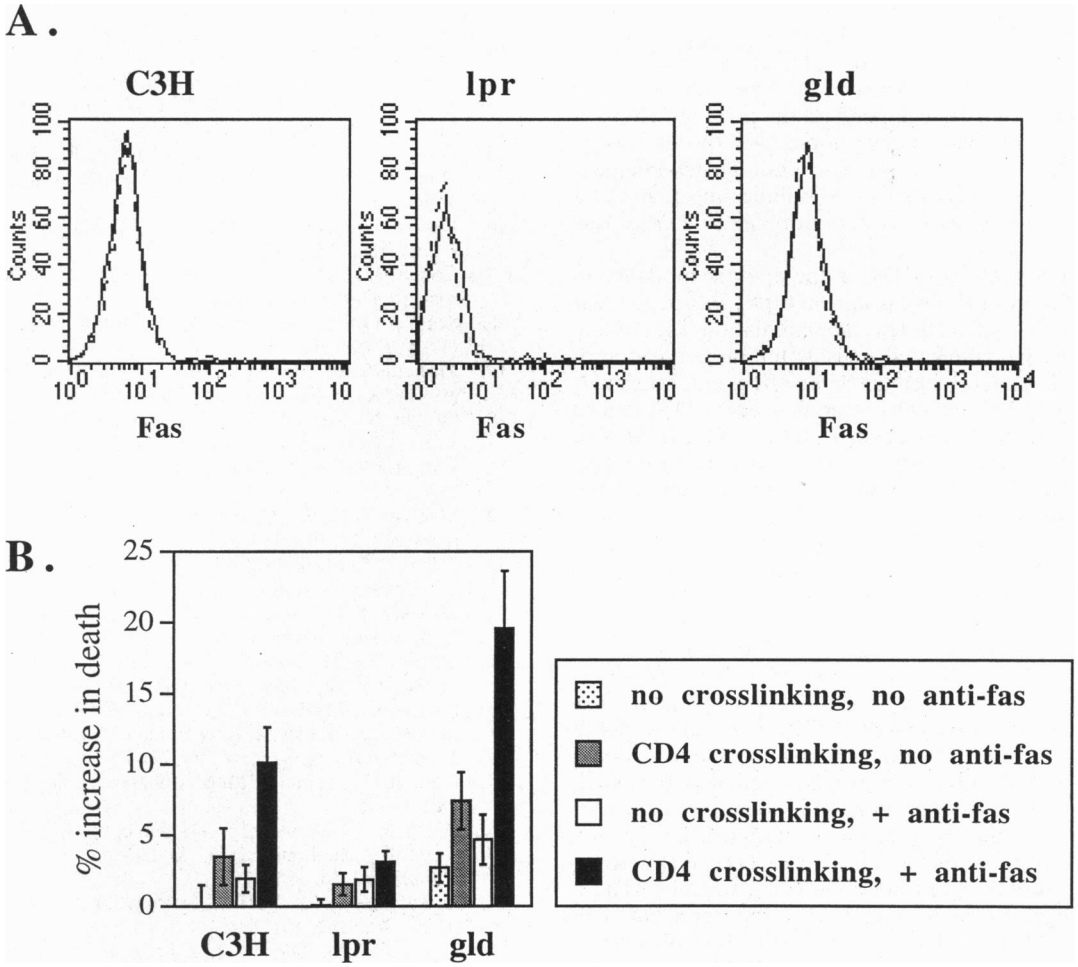

FIG. 5. CD4-induced Fas death function is independent of Fas surface expression levels. (*A*) Levels of Fas expression are identical on $CD4^+$ which have not (solid line) or have (dashed line) undergone CD4 cross-linking, after overnight incubation. (*B*) Cells from *A* were incubated an additional 18 hr, and then viability was determined. The increase in the percentage of dead cells during the 18-hr post-CD4 cross-linking (or control treatment) is shown.

triggered a mechanistically independent change in the cells' sensitivity to Fas-induced death. When $CD4^+$ T cells were cultured overnight in the absence of any treatment, they spontaneously upregulated Fas expression to levels similar to those induced by CD4 cross-linking. Subsequent CD4 cross-linking did not result in any further Fas upregulation (Fig. 5*A*). This allowed us to study the effect of CD4 cross-linking on Fas function, independent of its expression level, by using overnight cultures of $CD4^+$ splenocytes as our starting population. We found that despite having the same levels of surface Fas (Fig. 5*A*), only cultures treated with anti-CD4 displayed a Fas-dependent increase in cell death after a further 18-hr incubation (Fig. 5*B*). Thus, CD4 cross-linking alters the sensitivity of $CD4^+$ cells to Fas-induced death.

DISCUSSION

We have established that CD4 cross-linking increases the sensitivity of a subset of $CD4^+$ cells to Fas-induced death signals, as well as rapidly increasing cell surface Fas expression. It has been reported that after a 3-day incubation, CD4 ligation resulted in tumor necrosis factor-induced Fas upregulation on $CD4^+$ and $CD8^+$ cells (20). Our results demonstrate much earlier $CD4^+$ cell-specific effects of CD4 cross-linking. Both the rapid induction of Fas expression and Fas-mediated cell death occur selectively in $CD4^+$ T cells; $CD4^-$ cells in unfractionated spleen cultures are unaffected. Furthermore, experiments with isolated $CD4^+$ cells show that CD4-induced changes in Fas can occur independently of other cell types and their products. It seems clear, however, that only a subset of $CD4^+$ cells are affected, since we never observe 100% cell death in our cultures. We are presently investigating subsets based on cytokine production and memory markers.

HIV infection provides an interesting pathophysiological analogue to the system of *in vitro* CD4 cross-linking described here. HIV infects $CD4^+$ lymphocytes through the interaction of its surface glycoprotein, gp120, with CD4. Abnormally high Fas expression has been reported on the T cells of people infected with HIV (22, 23), and T cells from HIV patients are sensitive to Fas-induced apoptosis (24, 25), consistent with a CD4 cross-linking mechanism of Fas induction. *In vitro* and *in vivo* experiments have shown that binding of gp120 to CD4 results in apoptosis (4, 26–28). Interestingly, HIV-induced apoptotic cell death fails to occur in cells expressing low levels of CD4, or in $CD4^-$ cells transfected with CD4 missing its cytoplasmic tail, although these cells can become productively infected (27). gp120-triggered apoptosis is enhanced by the presence of the CD4-associated protein tyrosine kinase lck (27). In *lck*$^-$ mutant clones, gp120 was largely unable to induce apoptosis; in contrast, apoptosis was restored in clones reconstituted with functional lck, or with kinase-negative mutant lck.

The findings of HIV studies, considered together with the reports of an association between Fas signaling and lck-induced phosphorylation (29, 30), suggest that CD4-induced sensitivity to Fas-mediated death may be regulated by lck, perhaps independently of its kinase function. In fact, CD4

aggregation triggers changes in lck phosphorylation and function, variously reported as increased tyrosine phosphorylation at its negative regulatory site, Y505 (31), and at its activating (autophosphorylation) site, Y394 (32). Thus, the ultimate outcome of Fas ligation may depend on the prior intracellular distribution and/or phosphorylation state of lck. CD3-stimulated Jurkat cells, however, can undergo lck-independent, Fas-mediated apoptosis (33, 34), indicating that CD4-independent Fas-mediated cell death can also be lck-independent.

In vivo injection of anti-CD4, a therapeutic modality in autoimmune diseases (35, 36), results in rapid, Fas-dependent apoptosis of $CD4^+$ T cells in the circulation (5). When anti-CD3 antibody is coinjected, cells do not undergo apoptotic death (37). Thus, CD3 modulates the effect of CD4 signals on Fas function. In fact, Fas can costimulate anti-CD3-induced T-cell proliferation and interleukin secretion (38, 39). *In vivo*, the switch in Fas function from costimulation to death may therefore be controlled by the disappearance of antigen at the end of an immune response. CD4 cross-linking by major histocompatibility complex class II in the absence of sufficient antigenic signal could result in Fas-induced death, analogous to rescue from Fas-induced B-cell death by Ig signaling (40). A further analogy is that B cells are primed for Fas-mediated death by prior cross-linking of CD40, a costimulatory B-cell surface molecule (41, 42). *In vitro,* cocross-linking of CD3/TCR with CD4 results in enhanced activation (1, 2), whereas sequentially cross-linking CD4, then TCR, leads to cell death (3). As reported here, CD4 cross linking activates Fas death function, and cell death ensues when a ligand is provided, either exogenous anti-Fas antibody, as in our experiments, or endogenous FasL induced by TCR stimulation (3).

Our data demonstrate that a signal from CD4 can effectively render cells sensitive to Fas death-inducing function. These findings also suggest a mechanism for the reduced $CD4^+$ cell counts and shift from a Th1 to a Th0 (or Th2) phenotype observed during HIV infection (43–45), as the Th1 cells, more prone to FasL expression (46), undergo autocrine death as a consequence of gp120/CD4-induced Fas activation.

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